Time-Resolved Infrared ATR Measurements of Liposome Transport Kinetics in Human Keratinocyte Cultures and Skin Reveals a Dependence on Liposome Size and Phase State

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A novel *in vitro* method for studying the permeation kinetics of superficially applied liposomes or vesicles through layers of human skin or keratinocytes on a solid support is presented, employing attenuated total reflection infrared spectroscopy. The method is applied to investigate transport kinetics of unilamellar vesicles of dimyristoylphosphatidylcholine (DMPC) through cultured human keratinocyte layers and through human skin. We find a strong resemblance of the qualitative features of the permeation kinetics of small unilamellar DMPC vesicles for skin and keratinocytes. Detailed studies of the vesicles transport through keratinocyte layers show that DMPC vesicles with an average diameter of 55 nm can readily permeate through the layer at 37°C with a diffusion constant of $D = (4.0 \pm 0.8) \times 10^{-15}$ m^2/second, whereas larger vesicles of twice that diameter do not permeate at all. In contrast, liposomes containing a chemical permeation enhancer permeate through the layer significantly faster [$D = (7.0 \pm 0.5) \times 10^{-15}$ m^2/second] than the small DMPC vesicles despite their five-times-larger diameter. Moreover, the transport of the DMPC vesicles depends drastically on their phase state. No permeation was observed for small DMPC vesicles at a temperature of 10°C when the lipid is in the crystalline phase state.

Our results indicate that keratinocyte culture layers can pose a significant permeation barrier for vesicles. The permeation mechanism can be explained by diffusion of the vesicles through small pores and gaps in the layer, presumably driven by transdermal osmotic gradients. ***Key words: ATR FI-IR/diffusion constant/vesicle/permeation. J Invest Dermatol 105: 291–295, 1995***

The mechanism of liposome transport through the skin is of considerable interest for our basic understanding of the skin's barrier function as well as for many pharmaceutical and cosmetic applications (for a review, cf. [1]). It is generally assumed that the stratum corneum represents the main barrier for transdermal transport. The contribution of the other skin layers to the barrier function is not yet fully understood. Regarding the permeation of liposomes through the skin, there are two intriguing questions that give rise to considerable controversy in the literature: first, can liposomes permeate through the skin without losing their integrity, and, second, does the main pathway of permeation lead through the cells or is there a more efficient pathway through the gap junctions between the cells? Attempts to resolve these questions are hampered by the lack of suitable experimental setups that enable a sensitive measurement of the liposome permeation kinetics through both fully differentiated skin and its precursors (i.e., keratinocyte layers), and a simultaneous assessment of liposome integrity. So far, the main methods for studying skin-permeation processes are electron microscopy [2–4], diffusion cells [1], and tape-stripping methods [5]. The former two are *in vitro* methods, the latter two methods require the application of radioactive or fluorescent labeled compounds. A further *in vivo* method is the direct measurement of topically applied and liposomally encapsulated drugs in the bloodstream and its disposition in tissues [6–8]. All these methods have their particular merits but show certain drawbacks regarding their sensitivity and their time resolution for the measurement of permeation kinetics. Moreover, most of them require the application of radioactive labeled compounds.

A further limitation for a detailed analysis of liposome permeation through the skin is their comparatively slow permeation kinetics, owing to the thickness of the skin and to the very nature of the permeation mechanism. This, and the lack of suitable experimental methods may explain that most of the published work dealing with liposome permeation concentrate on their transport into the skin [1,6,9] but not through it. An advantageous model system for detailed liposome skin-permeation studies should exhibit the same basic transport mechanisms but on a shorter time scale.

In this work, we present a new *in vitro* method for measuring liposome transport through skin layers, based on time-resolved

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Abbreviations: ATR, attenuated total reflection; DMPC-d_{54}, 1,2 perdeuterio-dimyristoyl-sn-glycero-3-phosphocholine; FT-IR, Fourier transform–infrared.

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infrared attenuated total reflection (ATR) spectroscopy, which has the potential to meet the above-mentioned requirements for exploring the detailed permeation characteristics of liposomes. The application of infrared spectroscopy in skin research is well established [10] and has been demonstrated as particularly useful for the study of skin lipid-phase behavior [11,12], drug delivery [13], and skin hydration [14]. Using this method we concentrate on the transport of vesicles of 1,2 perdeuterio-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) through cultured human keratinocyte layers as a function of vesicle size and phase state and compare these results with the permeation characteristics obtained for fully differentiated skin. We suggest that human keratinocyte layers cultured on an ATR crystal as solid support represent an advantageous model system for skin-permeation studies. Although this system does not exhibit a stratum corneum–like fully differentiated skin, it may serve as a suitable model for studying the basic permeation mechanism and can approximate situations where the horny layer of intact skin is damaged, e.g., in the case of dermatitis.

MATERIALS AND METHODS

Materials The phospholipid 1,2 perdeuterio-dimyristoyl-sn-glycero-3-phosphocholine (DMPC-d_{54}) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL) and used without further purification. Its purity was checked by measuring the temperature and width of its phase transition by means of differential scanning calorimetry.

All lipid preparations were carried out in a 20-mM HEPES buffer at pH 7.0, adjusted with NaOH. If not otherwise indicated, the buffer contained 100 mM NaCl. Vesicles were prepared by treating an appropriate amount of DMPC-d_{54} in buffer (concentration 10 mg/ml) for 30 min at 30°C with a swelling procedure [15]. Small unilamellar vesicles were obtained by sonicating the swollen suspension with a titanium rod sonifier at 30°C until an optically transparent vesicle solution was obtained. The mean hydrodynamic diameter of these vesicles is d_h = 55 nm as determined by dynamic light scattering according to procedures described previously in detail [15].

Small unilamellar vesicle populations of different size were obtained employing the size extrusion technique [16]. The swollen DMPC-d_{54} suspension was 10 times extruded through polycarbonate filter membranes of either 50 or 200 nm pore size at 30°C. This procedure yielded two vesicle populations of d_h = 70 nm and d_h = 165 nm.

Preparation of Keratinocyte Cultures Keratinocyte cultures were prepared essentially according to Green *et al* [17]. Human skin biopsies from circumcision were trypsinized at 4°C for 18 h. Epidermal cells were removed by forceps, washed, and seeded on a mitomycin C–treated 3T3 feeder layer. Before reaching confluency keratinocytes were detached by Trypsin/ethylenediaminetetraacetic acid treatment, washed, and seeded on a silicon crystal that was embedded in agar (1% agar + DMEM). The medium was renewed every second day until cells reached confluency.

Light microscopic examination together with an immunohistochemical analysis of cytokeratines suggest that growth and differentiation of cultured cells are comparable to the epidermis.

FT-IR Measurements Infrared spectra were obtained with a Nicolet 60SXR Fourier transform infrared spectrometer equipped with a MCT detector and a horizontal ATR unit. Unless otherwise indicated, 500 scans were acquired at a resolution of 2 cm^{-1} and apodized by a Happ-Genzel function prior to Fourier transform. The background and reference spectra were recorded separately at the corresponding temperature and the latter was interactively subtracted from the sample spectra. The temperature of the sample was measured and controlled by an external water bath with an accuracy of ±0.1°C. Temperature and incubation time were controlled by a computer subsystem connected to the acquisition computer. A horizontally arranged 80 × 10 × 3 mm^3 silicon crystal was used as the ATR assembly inside a homebuilt teflon trough. The cell layer (keratinocyte culture or stripe of human skin) was placed at the upper side of the crystal and immersed in nutrition solution. A teflon frame was pressed on the layer to expose a part of the superficial side (2 cm^2) of the layer to ambient (non-occlusive) atmospheric conditions **(Fig 1)**.

A vesicle suspension of 200 μl (lipid concentration 10 mg/ml) was applied to this area and the FT-IR spectra acquisition and time measurement were started. Because the vesicles consist of chain perdeuterated DMPC-d_{54}, the absorption band of the CD_2 stretching vibration (2050–2250 cm^{-1}) can be observed separately from all other signals. Owing to the thickness of the cultured skin (≈20 μm), which is large compared to the penetration depth of the IR evanescent field into the bottom layer (dermal side) of the keratinocyte culture or skin, no signals in that wavenumber region can be

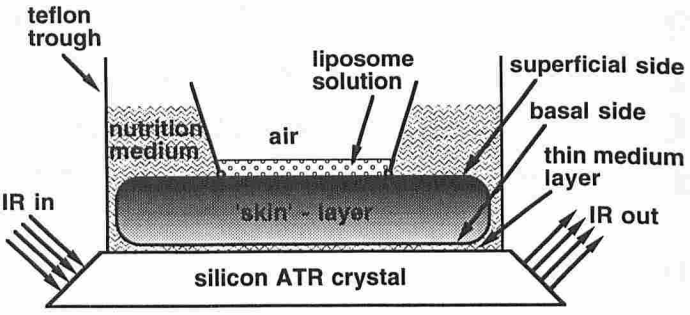

Figure 1. The infrared ATR technique applied for measuring permeation through "skin" layers. Schematic representation of the experimental setup used for the measurements.

observed in the initial stage of the experiment, i.e., right after the superficial application of the suspension. After this, transport of vesicles through the keratinocyte layer give rise to a FT-IR signal from the DMPC-d_{54} and its intensity increases with time. The evanescent IR field ensures that only those vesicles that managed to pass through at least 95% of the total layer thickness can significantly contribute to the signal. Intensity normalization is achieved by using the ratio of the measured absorption signal to that obtained from a separate measurement with the same amount of vesicles (200 μl) placed directly on the ATR plate (i.e., without any skin layer). Plotting the intensity of the CD_2 stretching vibration signal versus time gives the transport kinetics of the liposomes, which can be fitted by a single exponential according to $I(t) = 1 - \exp(-kt)$, giving the rate constant k of the permeation process. Under certain approximations this rate constant can be related to a diffusion coefficient D of the vesicles in the layer (cf. *Discussion*).

RESULTS

Vesicle Permeation in Keratinocyte Cultures Depends on the Phase State These measurements were performed using the same keratinocyte culture sample at two temperatures after the superficial application of highly sonicated DMPC-d_{54} vesicles. The mean hydrodynamic diameter of the vesicles was d_h = 55 nm as measured by dynamic light scattering. The first measurement was done at 37°C with the vesicles in the fluid L_α phase. Selected spectra of the CD_2 stretching vibrations for different times after the small unilamellar vesicle application are shown in **Fig 2**. The permeation kinetics obtained are represented in **Fig 3** and the single exponential fit to the data gives a rate constant $k = 6.6 \times 10^{-3}$

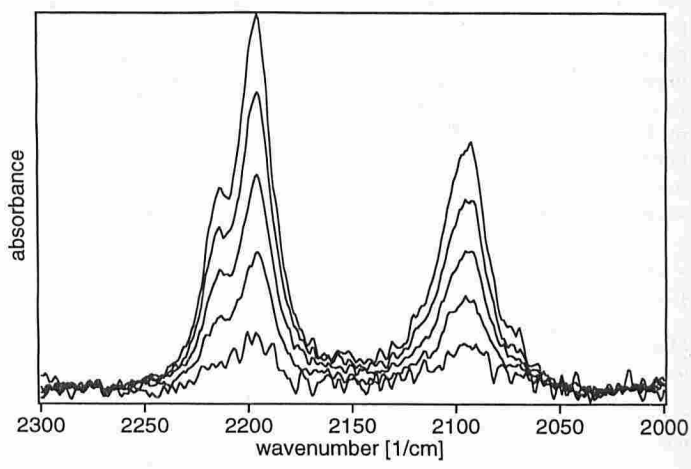

Figure 2. Very small DMPC vesicles can permeate keratinocyte layers. Time course of the FT-IR spectrum (asymmetric and symmetric stretching vibration region of the deuterio-methylene groups) arising from DMPC-d_{54} vesicles permeating a layer of cultured keratinocytes, measured using the setup shown in **Fig 1**. The bottom spectrum was obtained after 30 min, the upper spectrum after 450 min.

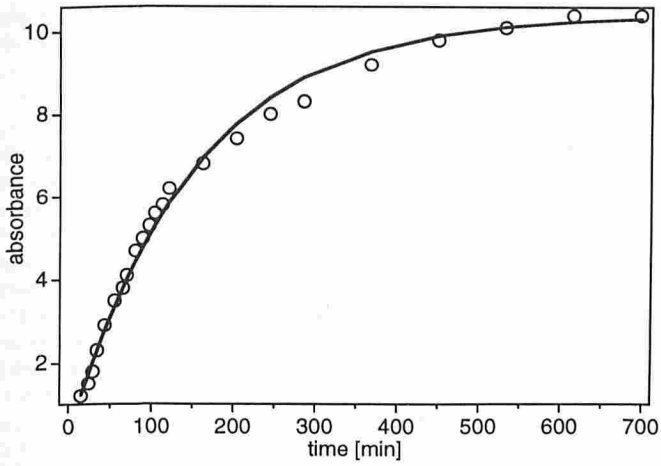

Figure 3. Permeation of very small DMPC vesicles through keratinocyte layers is an exponential function of time. Plot of the symmetric stretching vibration intensity (signal at 2095 cm^{-1} in **Fig 2**) versus time for DMPC-d_{54} sonicated vesicles permeating cultured keratinocyte layers. Data were obtained by a time resolved FT-IR measurement and the *solid line* represents a fit to a single exponential giving $k = 6.6 \times 10^{-3}$ min^{-1}.

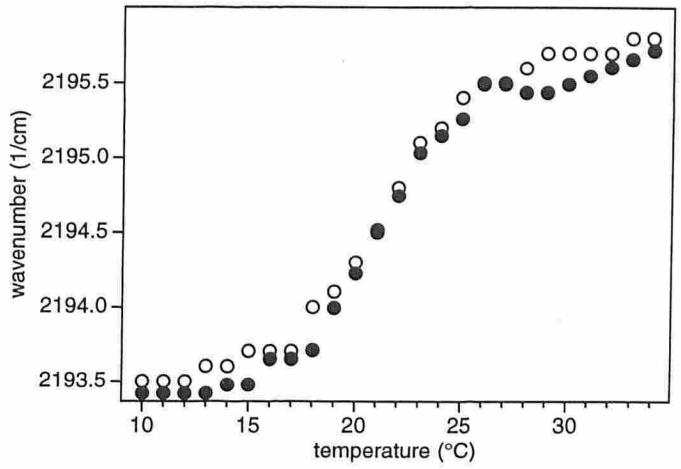

Figure 4. Phase-transition behavior of the vesicles is not altered by the permeation process. FT-IR ATR data of the temperature dependence of the CD_2 symmetric stretching vibration frequency of sonicated vesicles prior to (●) and after (○) permeation through a keratinocyte layer.

min^{-1}. The data indicate that 50% of the vesicles passed the layer within a permeation halftime $t_{1/2} \approx 100$ min.

The measurement was stopped after 12 h when equilibrium was reached and no further significant increase of the IR absorption intensity was observed.

The ATR assembly with the keratinocyte culture was then cooled down to 10°C where the DMPC-d_{54} vesicles are in the crystalline L_β phase. New vesicles (same amount as above) were transferred from a reservoir, which was kept at 10°C, to the layer surface (same site and area as for the first superficial application at 37°C) and the time resolved FT-IR measurement was resumed. Interestingly, the intensity of the signals in **Fig 2** did not show any further increase over time comparable to the first permeation experiment. This indicates that (if at all) only a very low amount of DMPC-d_{54} vesicles, well below the detection sensitivity of our setup, can pass the layer at this temperature. However, heating up the system to 37°C again after 12 h caused an increase of the signal intensity at the same rate as observed in the first experiment at this temperature (data not shown). To ensure that the observed blocking of vesicles permeation at low temperature is not caused by a presaturation of the layer from the previous experiment at 37°C, we repeated the 10°C measurement with a new keratinocyte layer sample with the low-temperature measurement being the first permeation experiment done with this sample. Even an incubation of the DMPC-d_{54} vesicles for up to 24 h at 10°C did not give rise to any signal intensity in the 2050–2250 cm^{-1} wavenumber region.

Phase Transition Behavior of the Vesicles Is Not Altered by the Permeation Process The temperature and width of the phase transition of a vesicle consisting of a synthetic saturated phospholipid is a measure for its purity and is effected by its curvature. Because the setup of our experiment is essentially sensitive to vesicles, which permeated through the skin into the aqueous gap between the silicon surface of the ATR crystal and the basal side, the phase transition can be measured after the end of the permeation experiment by measuring the frequency shift of the CD_2 stretching vibration as a function of temperature. A plot of the frequency versus temperature gives the well-known sigmoid shaped curve [18] as shown in **Fig 4**. In a separate experiment, the frequency dependence was obtained for the sonicated vesicles alone right after their preparation, which is also presented in **Fig 4**. It is interesting to note that there is no significant difference of the phase-transition behavior of the vesicles prior to and after the permeation through the skin.

Vesicles Permeation Is Size Dependent In a second series of experiments, we tested the effect of vesicle size for the permeation process. This was achieved by preparing large DMPC-d_{54} vesicles (d_h = 165 nm as measured by dynamic light scattering) by the size-extrusion technique (cf. *Materials and Methods*).

Applying these vesicles superficially (same concentration as in the above experiments) to a keratinocyte layer at T = 37°C did not give rise to any signal in the 2050–2250 cm^{-1} range over a 12-h time period. Hence, none of the larger vesicles can permeate the skin.

In contrast, the application of smaller vesicles (d_h = 70 nm) prepared by size extrusion under identical conditions to the same skin sample, gives rise to a signal with a similar time course as observed for the sonicated vesicles (d_h = 55 nm) at 37°C (cf. **Fig 3**).

Chemical Permeation Enhancement Enables Permeation of Large Vesicles Here we compared the permeation of small sonicated vesicles as used in the first experiment (d_h = 55 nm) with that of liposome-like structures called transfersomes. The latter is specially designed to facilitate its transport through the skin by the addition of chemical permeation enhancers to the phospholipid [19,20]. The size of the transfersomes was d_h = 250 nm. A new keratinocyte culture sample was used, and first the permeation of the sonicated DMPC-d_{54} vesicles was measured at 37°C. The time course of the signal is represented in **Fig 5**, and the fit of the data (full line) gave $k = 6.4 \times 10^{-3}$ min^{-1} in good agreement with the permeation rate constant obtained above **(Fig 3)**. After 12 h a transfersome solution (same concentration as the sonicated vesicles) was applied superficially under the same conditions. To distinguish DMPC-d_{54} vesicles and transfersome permeation, the latter was obtained by following the change of the intensity of the CH_2 vibrations (2750–2950 cm^{-1}) as a function of time. Note that the transfersomes are a mixture of DMPC with fatty acids and other adjuvants that were all nondeuterated. As can be seen from **Fig 5**, the tranfersome permeation is significantly faster than that of the sonicated vesicles, giving a permeation rate constant from the single exponential fit to the data (full line) of $k = 1.0 \times 10^{-2}$ min^{-1}. Thus, the transport of the transfersomes through the keratinocyte culture is nearly twice as fast as that of the vesicles.

Human Skin Exhibits a Qualitatively Similar Permeation Characteristics for Liposomes as Keratinocyte Cultures For this experiment, the keratinocyte culture on the ATR crystal

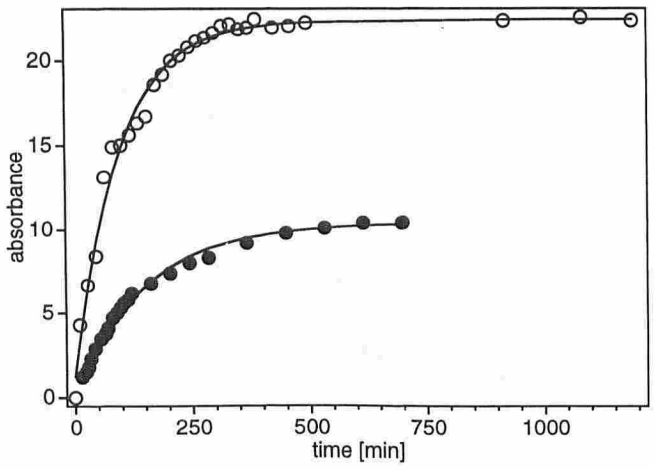

Figure 5. Transfersomes permeate faster than vesicles. Plot of the absorption intensity of the CD_2 symmetric stretching vibration band of DMPC-d_{54} sonicated vesicles (●) and of the CH_2 symmetric stretching vibration band of transfersomes (○) after permeation through cultured skin at 37°C. Data sets were obtained by time-resolved FT-IR employing the ATR setup shown in **Fig 1**. The *solid lines* represents fits of the data to a single exponential with $k = 6.4 \times 10^{-3}$ min^{-1} (vesicles) and $k = 1.0 \times 10^{-2}$ min^{-1} (transfersomes).

was replaced by a 5-cm^2 stripe of human skin. This skin was obtained post mortem as full-thickness skin from the (inner) thigh of a female donor (age 70 years). The basal side faced the ATR crystal surface and was immersed in nutrition solution while the superficial side was exposed to ambient conditions. The same concentration of small sonicated vesicles as used for the keratinocyte measurements shown in **Fig 3** was applied to the skin surface at 37°C, and the transport was measured analogously. The result is shown in **Fig 6**. It is obvious that this permeation is significantly slower than measured for keratinocyte cultures. No halftime for the permeation can be given because no equilibrium was reached during the time of the experiment (12 h). It should be noted, however, that the result agrees qualitatively with that obtained for the keratinocyte culture (also shown in **Fig 6**), though the time

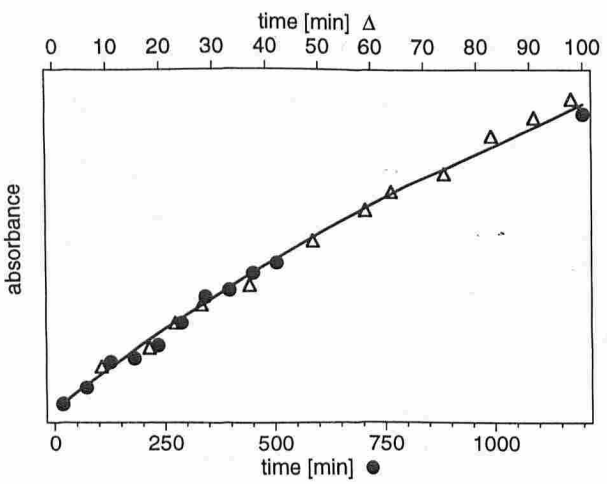

Figure 6. Comparison of permeation characteristics for keratinocyte layers and intact human skin. FT-IR absorption data versus time for sonicated DMPC-d_{54} vesicles permeating intact human skin (●) and keratinocyte layers (Δ). Note the different abcisses for the two kinetics in the representation. Data fits give $k = 6.5 \times 10^{-4}$ min^{-1} and $k = 6.6 \times 10^{-3}$ min^{-1} for human skin and keratinocytes (*solid line*).

scale is different. Both processes can be fitted to single exponentials, giving $k = 6.5 \times 10^{-4}$ min^{-1} for human skin in comparison to $k = 6.6 \times 10^{-3}$ min^{-1} as reported above for keratinocytes.

DISCUSSION

Permeability Barrier and Vesicles Integrity It is generally assumed that the stratum corneum of intact human skin represents its main permeability barrier for vesicles and liposomes applied on the skin surface [20,21]. Keratinocyte cultures as used in our experiments do not exhibit a stratum corneum despite their high degree of differentiation (cf. *Materials and Methods*). Nevertheless, our results indicate that even the keratinocyte layer poses a significant permeability barrier for vesicles. Moreover, the similarity of the (initial) liposome transport kinetics measured for keratinocyte cultures and for intact human skin **(Fig 6)** suggests that both systems exhibit a qualitatively similar behavior, but on a different time scale. The differences in thickness by more than one order of magnitude between skin and keratinocytes may account for this.

One of the most intriguing questions is about structural changes of the vesicles owing to the transport, i.e., whether the vesicles remain morphologically intact or undergo fragmentation or decomposition upon passing through the keratinocyte layer. The infrared ATR technique measures an intensity arising from the IR absorption of the lipids within the detection range of the evanescent field on the ATR crystal, it cannot distinguish its specific environment. However, such a distinction can be achieved by comparing the phase-transition behavior of the vesicles prior to and after their transport through the layer. Vesicles, which permeate through the keratinocyte culture without significant changes of their morphology, will exhibit a phase-transition behavior similar to the control. On the other side, should the vesicles decompose on their way through the keratinocyte culture and their lipids become part of the keratinocyte wall or interior, their phase transition would be rendered undetectable owing to the mixing with lipids of the host cell. The sigmoid-shaped temperature dependence of the CD_2 stretching vibration frequency as shown in **Fig 4** does not differ significantly from that of the vesicles prior to permeation. This finding provides strong evidence that the vesicles retain their integrity upon passing through the keratinocyte layer.

Permeation Mechanism and the Liposomes Diffusion Constant At least two pathways have been suggested along which vesicles may pass through the skin. Permeation by transport (active or passive) directly through the cells by means of endocytosis and exocytosis is well established. Recently, a more direct pathway along the pores and gaps in the skin has been suggested. The latter does not involve any contact of the vesicles with the cytoplasm, and its driving force are transdermal osmotic gradients and hydration forces [20,21]. These gradients are believed to arise due to the application of the vesicle solution on the layer surface under non-occlusive conditions. Because the dimensions of pores and gaps between cells in the skin are presumably small (nm range), this pathway must be very sensitive to the size of the vesicles.

Our results indicate a drastic dependence of the vesicle permeation on the size of the vesicles. Although 70-nm vesicles can readily pass the keratinocyte layer **(Fig 3)**, vesicles of about twice that size are blocked. This is a result that is hard to explain on the basis of active transport by endo- and exocytosis. However, it is quite conceivable by assuming that the vesicles permeate through the pores and gaps in the keratinocyte layer. The result would indicate that the minimum diameter of the channels through the layer is between 70 and 150 nm.

On the other hand, vesicles in the fluid L_α phase exhibit a certain deformability, and thus are enabled to pass pores of significantly smaller size than their own diameter. Hence, the pore-size limit given above might be an overestimate. Our experiments strongly support this because we found a dependence of the permeation on the vesicle phase state. The major difference between vesicles in the fluid L_α and the crystalline L_β phase state is their rigidity with respect to deformations of the bilayer. L_α phase vesicles exhibit an

elasticity constant K_c that is only one order of magnitude above the thermic energy [22], and thus can readily change their shape. In contrast, L_β phase vesicles are virtually undeformable hard spheres, owing to their several magnitudes higher K_c. The latter can pass pores only when they are larger in size than the vesicles. Hence, this finding puts the limit for the pore size in the keratinocyte culture in the range of less than 70 nm.

Another explanation for the temperature dependence of the vesicle permeation in terms of an active transport mechanism through the cells could be the cessation of the active transport below a certain temperature. It is well established that a critical temperature exists for exo- and endocytosis below which these processes come to a halt. However, this would not explain the vesicle size dependence on the permeation as reported above.

Further support for our interpretation of the vesicle permeation in terms of a pore diffusion driven by transdermal osmotic gradients and hydration forces comes from the transfersomes measurements. They can pass through the layer more readily than vesicles despite their five times larger diameter. Transfersomes are liposome-like structures specially designed for an extreme softness of their bilayer, thus permitting highest deformability upon pore diffusion.

Assuming the validity of the pore-diffusion model and a homogeneous distribution of pores over the layer, we can use an analytical solution of Fick's second law for expressing the vesicles permeation through the layer in terms of a diffusion coefficient D according to $I(t) = 1 - \exp(-kt) = 1 - \exp(-\pi^2 Dt/d^2)$.

Here I(t) is the (normalized) IR absorption intensity and d is the thickness of the layer which is 20 ± 2 μm in our experiments as determined by light microscopy. For the sonicated vesicles with $k = 6.6 \times 10^{-3}$ min^{-1} **(Fig 3)** we obtain $D = (4.0 \pm 0.8) \times 10^{-15}$ m^2/second, whereas for the transfersomes with $k = 1.0 \times 10^{-2}$ min^{-1} **(Fig 5)** a value $D = (7.0 \pm 0.5) \times 10^{-15}$ m^2/second is obtained.

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